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Effects of Daidzein on mRNA Expression of Gonadotropin Receptors and P450 Aromatase in Ovarian Follicles of White Silky Fowls

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ABSTRACT: Effects of daidzein on expression of mRNAs of gonadotropin receptors (FSHR, LHR) and P450 aromatase (P450arom) were evaluated in ovarian follicles of white silky fowls. The hens were 13 months old in the post-peak period of egg laying and were randomly allocated as control and daidzein-treated groups, with daidzein supplemented to the basal diet at 10 mg/kg for 7 consecutive weeks. The mRNA expression of related genes was measured by semi-quantitative RT-PCR in the granulosa layers of the preovulatory follicle (PRF: F1, F2 …) and follicular layers of the small yellow follicle (SYF), large white follicle (LWF) and atretic follicle (ATF). Results showed that daidzein supplementation significantly increased the number of SYF and LWF ($p<0.05$). The relative abundance of the FSHR mRNA decreased in the granulosa layers from F3 to F1, but LHR mRNA displayed opposite developmental changes. P450arom mRNA was highest in the SYF, but was very low in the granulosa layers after follicles finished selection. Treatment with daidzein resulted in increased mRNA expression of FSHR in F3 granulosa layer, LHR in granulosa layers of F3 to F1 and P450arom in LWF ($p<0.05$). These results indicated that dietary supplementation of daidzein up-regulated mRNA expression of gonadotropin receptors and P450arom to improve the development of preovulatory follicles in white silky fowls after the peak-laying period. (**Key Words:** Daidzein, Gonadotropin Receptor, P450 Aromatase, White Silky Fowl, Follicle)

INTRODUCTION

White silky fowl is an extraordinary fowl indigenous to China. They are mild, short, with a small and long head but short neck and can be easily distinguished from other chickens. The eggs and meat of white silky fowl are well known in the orient for their abundant contents of unsaturated fatty acids, vitamins, calcium and potassium compared to common chickens (He, 2003; Toyosaki and Koketsu, 2004). They have been credited with Chinese traditional medicinal and health-promoting values for thousands of years. As the reproductive capacity of white silky fowl is rather low, it is very necessary to investigate the influencing factors of reproduction including hormones, growth factors, cytokines and correlative gene expression.

Daidzein belongs to the most common group of isoflavones found in plants such as soybeans, clover, and bluegrass and has been studied extensively for possible beneficial biological activities, including estrogen-like and estrogen-independent effects (Wang et al., 2002; Choi et al., 2006; Liu et al., 2006). Picherit et al. (2000) reported that daidzein could bind to rat uterus estrogen receptor (ER), inducing either anti-estrogenic or very weak estrogenic effects (depending on the experimental conditions) and *in vitro* uterine responsiveness to oxytocin and prostaglandin $F_{2\alpha}$ or luprostiol. However, the influence of daidzein on reproductive performance of domestic animals (especially the local poultry) is relatively scarce.

During growth and development, ovarian follicles undertake a series of complex biochemical and physiological changes that include gonadotropin receptor expression, steroid biosynthesis, cell proliferation and differentiation. Among these changes the expression of gonadotropin receptors (FSHR, LHR) and P450 aromatase (P450arom) plays very important roles in inducing follicular development (Nitta et al., 1991; Zhang et al., 1997). Estrogen had a stimulatory effect on the expression of ER and gonadotropin (Ing and Tornesi, 1997), and the estrogen-ER interactions may be involved in follicular growth and the local control of steroidogenesis in the follicles (Yoshimura et al., 1995). Our previous studies revealed that daidzein stimulated ovarian germ cell proliferation in chicken embryos (Liu et al., 2006). However, the precise mechanisms of daidzein on regulating mRNA expression of FSHR, LHR and P450arom are not

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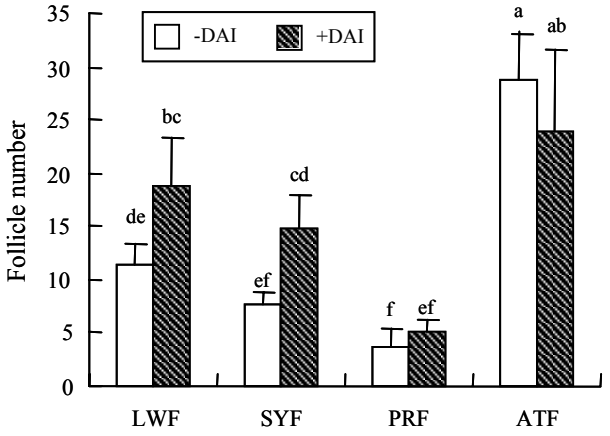

Figure 1. Effects of daidzein on follicle development in white silky fowls. Values are the mean±SD. Bars with different superscripts are statistically different ($p<0.05$, n = 8). LWF: large white follicle, SYF: small yellow follicle, PRF: preovulatory follicle, ATF: atretic follicle.

well understood. The objective of this study was to examine the effects of daidzein on follicle development and mRNA expression of gonadotropin receptors and P450arom in local white silky fowl.

MATERIALS AND METHODS

Animals

In this experiment, 16 white silky fowl (13 months old) in the post-peak period of egg laying were randomly allocated as control and daidzein-treated groups, with daidzein supplemented to the basal diet at the level of 10 mg/kg, for a period of 7 weeks. Hens were given free access to water and feed under natural light cycles in late spring.

Collection of ovarian follicles

The white silky fowls were sacrificed by cervical bleeding post anesthesia. The granulosa layers of preovulatory (PRF: F3-F1) and atretic follicles (ATF) were separated and snap frozen in liquid nitrogen. Whole small yellow (SYF) and large white follicles (LWF) were frozen after removal of the yolk.

Total RNA isolation

Total RNA of granulosa layers of F3-F1, the follicular layers of SYFs and LWFs were extracted by Trizol reagent (GIBCO-BRL, USA) according to the manufacturer's instruction. RNA concentration and purity were determined with a spectrophotometer by calculating the ratio of optical density at wavelengths of 260 and 280 nm. The ratios were between 1.8 and 2.0.

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA (1 μg) was denatured at 70°C for 5 min with 0.5 μg Oligo $(dT)_{18}$ primer (Bio-Synthesis, USA) and reverse-transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, USA) in a 20-μl reaction. The reaction mix was incubated for 60 min at 42°C and inactivated by heating at 70°C for 15 min. Two microliters of the reverse-transcribed product was subsequently used for PCR amplification. For PCR, 0.5 μM specific primers for respective target genes and 0.5 units of Taq DNA polymerase (Madison, WI, USA) in a total volume of 50 μl were used. For PCR amplifications, the following primer pairs of FSHR, LHR, P450arom and β-actin (synthesized by Shanghai Sangon Co. Ltd) were used, respectively: forward primer 5'-AGA AGG CCA ACA ACC TCG TG-3' and reverse primer 5'-ACAGCA ATG GCT AGG ATA GGT-3'; forward primer 5'-CTC AGG CGG ATA CAC AAC GA-3' and reverse primer 5'-TCA GAA CAG CTT CCA GCA GG-3'; forward primer 5'-TGA ACA GAA CTT TGA GCT-3' and reverse primer 5'-ATG AAG GCA TTC TTA AGT-3'; forward primer 5'-ACG TCG CAC TGG ATT TCG AG-3' and reverse primer 5'-TGT CAG CAA TGC CAG GGT AC-3'. Each target gene was co-amplified with β-actin in the same reaction. By adjusting the ratio of β-actin primers to those of the target genes, the overall PCR amplification efficiency of β-actin can be adjusted to the level comparable to the target genes. The respective amplification programs of FSHR, LHR and P450arom were as follows: 94°C, 30 s; 54°C, 60 s; 72°C, 60 s; 30 cycles; 94°C, 30 s; 54°C, 60 s; 72°C, 60 s; 23 cycles; 94°C, 30 s; 60°C, 60 s; 72°C, 60 s; 35 cycles. All samples were included in the same run of RT-PCR and repeated at least three times.

Quantitation of PCR products

PCR product (10 μl) was analyzed by electrophoresis on 1.2% agarose gel. The gel was stained with ethidium bromide. The net intensities of individual bands were measured using Image Master VDS Software (Pharmacia Biotech, Sweden). The ratios of the intensities of target genes to β-actin represented the relative mRNA abundance of the target genes. The average level of three repeats was used for statistical analysis.

Statistical analysis

All data were expressed as the mean±SD and analyzed by ANOVA and Duncan's multiple range tests using the SAS 9.0 software. $p<0.05$ was considered significantly different.

RESULTS

Effects of daidzein on follicle numbers

There was no significant difference in the number of

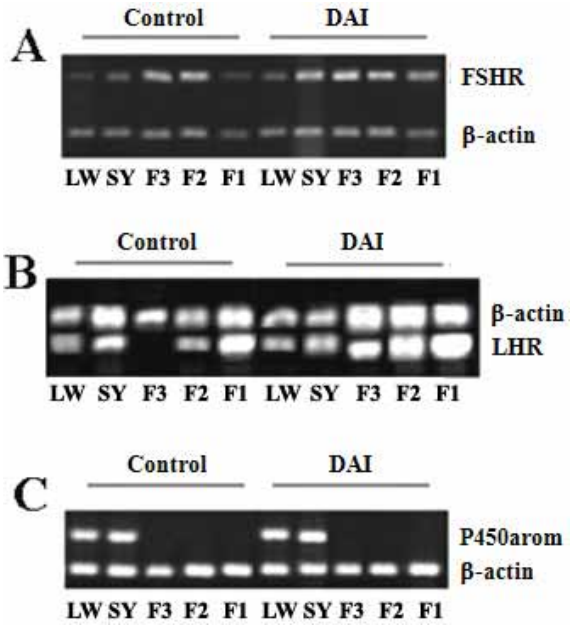

Figure 2. Effects of daidzein on expression of FSHR, LHR and P450arom mRNAs in ovarian follicles of white silky fowls. (A) to (C) represent electrophoresis of RT-PCR products for FSHR (521 bp), LHR (193 bp) and P450arom (486 bp) versus β-actin (282 bp) mRNAs, respectively. LWF: large white follicle, SYF: small yellow follicle, F3-F1: granulosa layers of F3-F1.

PRF between the control and daidzein-treated white silky fowl, but there was a marked increase in the number of SYF and LWF after daidzein treatment over the experimental period of 7 weeks (Figure 1).

Expression of FSHR mRNA

All RT-PCR products were cloned and sequenced. Their homology was ≥99% compared with respective genes (*Gallus gallus*) reported in GenBank. In the granulosa layers of the preovulatory follicles, relative abundance of FSHR mRNA expression showed the highest level in the F3, decreased in the granulosa layers of F2, and remained low in F1. Meanwhile, the FSHR mRNA levels of SYF and LWF were lower compared with that of F3 granulosa layer. After treatment with daidzein supplemented in the feedstuff, the FSHR mRNA manifested an increased expression in the F3 follicle (Figures 2A and 3; $p<0.05$).

Expression of LHR mRNA

In contrast to FSHR, mRNA expression in the granulosa layers of PRFs, LHR mRNA abundance showed the lowest level in the granulosa layer of F3, increased in the granulosa layers of F2 and increased markedly in the granulosa layer of F1 to reach the highest expression. However, LHR mRNA abundance remained low in both SYF and LWF. In the daidzein-treated group, LHR mRNA expression in the granulosa layers of PRFs was significantly higher than that

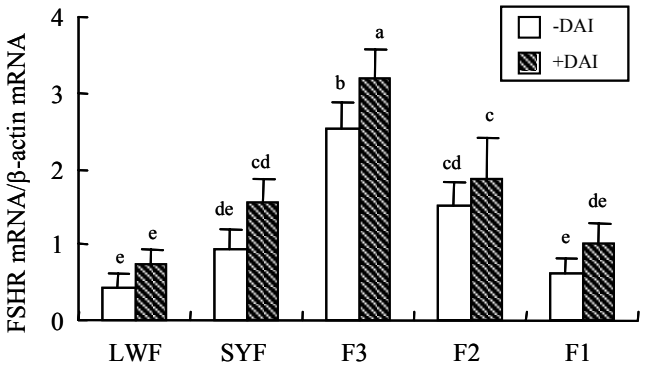

Figure 3. Effects of daidzein on expression of FSHR mRNA in ovarian follicles of white silky fowls. FSHR mRNA levels are expressed as arbitrary units relative to β-actin mRNA. Values are the mean±SD (n = 4). Bars with different superscripts are statistically different.

of the control group (Figures 2B and 4; $p<0.05$). However, no obvious changes of LHR mRNA level were found in daidzein-treated SYF and LWF follicles compared with the control.

Expression of P450arom mRNA

P450arom mRNA levels remained very low in the granulosa layers of PRFs. Nevertheless, the relative abundance of P450arom mRNA in SYF and LWF was higher than that of PRFs. Meanwhile, compared with the control group, the P450arom mRNA displayed an increased expression in LWF of the daidzein-treated hens (Figures 2C and 5; $p<0.05$).

DISCUSSION

Hen ovary is an excellent model to study follicular selection because the stage of follicular development can easily be determined by the size of the follicle. The ovary of the laying hen contains five to eight large yolk-filled preovulatory follicles. These large follicles are arranged in a hierarchy according to size, with the largest follicle designated as the F1 follicle and the second largest designated as the F2 follicle and so on for the other follicles. After ovulation of the F1 follicle, the F2 follicle becomes the new F1 follicle, succeeding follicles each advance one place in the hierarchy and an additional follicle is recruited from the pool of small nonhierarchical follicles. The small nonhierarchical follicles consist of a pool of SYFs that are about 5-10 mm in diameter and a pool of white follicles that are less than 5 mm in diameter. A single follicle is selected each day from the pool of SYFs to join the exclusive group of PRF destined for ovulation. The growth and development of follicles undertake a series of complex biochemical and physiological changes. However, the precise mechanisms that regulate the intricate follicular hierarchy in avian

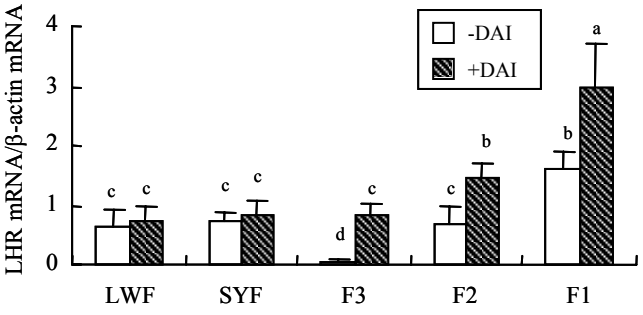

Figure 4. Effects of daidzein on expression of LHR mRNA in ovarian follicles of white silky fowls. LHR mRNA levels are expressed as arbitrary units relative to β-actin mRNA. Values are the mean±SD (n = 4). Bars with different superscripts are statistically different.

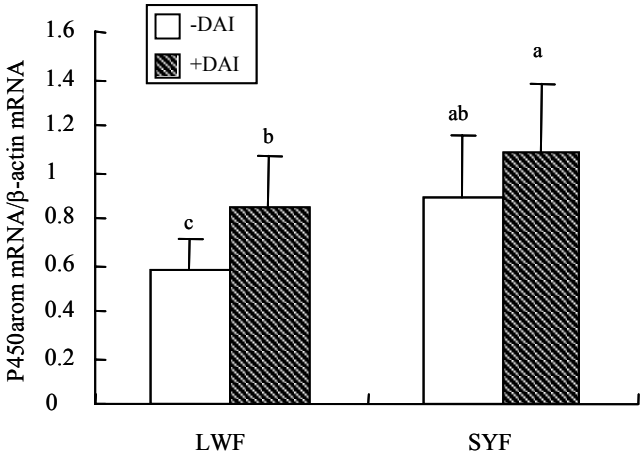

Figure 5. Effects of daidzein on expression of P450arom mRNA in ovarian follicles of white silky fowls. P450arom mRNA levels are expressed as arbitrary units relative to β-actin mRNA. Values are the mean±SD (n = 4). Bars with different superscripts are statistically different.

species are not well understood.

The present study demonstrated the promoting effects of daidzein on follicle development and mRNA expression of three genes closely related with follicle development in white silky fowl. We found that daidzein treatment markedly increased the numbers of SYF and LWF compared with the control in white silky fowl after peak laying. Daidzein up-regulated the mRNA expression of FSHR in F3 granulosa layer and LHR in granulosa layers of F3 to F1. Moreover, daidzein treatment increased the P450arom mRNA expression of LWF. Controversial positive and negative effects of daidzein on reproduction have been reported. Earlier studies suggest that phytoestrogens may cause follicular abnormalities, infertility in animals and decline of egg laying (Leopold et al., 1976). However, our previous studies revealed that phytoestrogens stimulated germ cell proliferation in chicken embryonic ovary (Liu et al., 2006; Liu and Zhang, 2006). Feeding daidzein to Shaoxing ducks significantly improved the laying performance (Zhao et al., 2004).

Gonadotropins are the primary regulators of follicular growth and ovulation. FSH is responsible for follicular recruitment and growth of the smaller follicles. The primary target for LH is the granulosa layer of the larger PRFs (Calvo and Bahr, 1983). Gonadotropins exert their actions by binding to specific G protein-coupled gonadotropin receptors. Many studies on FSHR and LHR gene expression have been conducted in mammals, but relatively less work in avian species has been reported. In the present study, we found that the highest expression of FSHR mRNA in the granulosa layer of F3, the intermediate expression in F2 and the lowest expression in F1. The FSHR mRNA level of SYF was higher than that of LWF. These results were consistent with previous reports that the granulosa layer of SYF and F6 to F3 are the primary targets for FSH (Zhang et al., 1997; Calvo and Bahr, 1983). The marked expression of LHR mRNA in the granulosa layer of F1-F2 and lower expression in the F3 may be important for LHR protein synthesis and subsequent increased progesterone production in F1 destined for ovulation. The LHR mRNA level of SYF was higher than that of the granulosa layer of F3. These results were in agreement with reports that LH promotes progesterone secretion by the granulosa cells of F3 to F1 as the follicles approach ovulation (Hammond et al., 1981). Meanwhile, expression of FSHR and LHR mRNAs in daidzein-treated groups was increased, which suggests that daidzein up-regulated the expression of FSHR and LHR mRNA in stage-related follicles. Estrogen stimulates the proliferation of granulosa cells in follicles and the expression of gonadotropin (Ing and Tornesi, 1997). Furthermore, estrogen synergizes with FSH and exogenous cAMP to increase the number of FSHR (Richards, 1980). According to these studies, the effect of daidzein on the up-regulated expression of FSHR and LHR mRNAs might be through the direct estrogenic actions.

P450arom catalyzes the conversion of androgen to estrogen and represents a rate-limiting enzyme in estrogen biosynthesis. Thus, the regulation of P450arom mRNA expression is important for estrogen production, subsequently for follicle development and oocyte maturation (McPhaul et al., 1988). Contrary to mammalian species, P450arom mRNA level remained very low in the granulosa layers of hen PRFs. The absence of P450arom mRNA in granulosa layers is consistent with the findings that aromatization of estrogen from androgen is limited to the theca layer of PRFs in avian follicles (Nitta et al., 1991; Porter et al., 1991), and the resultant increase of estrogen secretion from the prehierarchical follicles is important in supporting the reproductive system of the laying hens. Furthermore, treatment with daidzein increased the P450arom mRNA expression of LWF. Sakimura et al. (2002) reported that estrogen treatment stimulated ERα and

P450arom mRNA expression in chicken gonads at the early stage of embryonic development. Moreover, ER immunoreactions were demonstrated in chicken white follicles and outer theca externa and granulosa cells of the F3 follicle (Yoshimura et al., 1995). Because of the structural similarity to natural estrogens, daidzein may exert estrogenic activities in animals, humans and cultured cells. The increase of P450arom expression after daidzein treatment may be due to the estrogenic actions of daidzein on P450arom mRNA expression in white silky fowl immature follicles.

In summary, developmental stage-related differences in mRNA expression of gonadotropin receptors and P450arom was revealed in the ovarian follicles of white silky fowls. Dietary supplementation of daidzein improved the follicle development via up-regulated mRNA expression of gonadotropin receptors and P450arom. These results indicated that daidzein, as a dietary supplement, may promote follicle development involving increased mRNA expression of three genes closely related with follicle development in white silky fowls after the peak-laying period through estrogenic effects.

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